Can behaviour impede evolution? Persistence of singing effort after morphological song loss in crickets

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10   Evolutionary loss of sexual signals is widespread. Examining the consequences for

11   behaviours associated with such signals can provide insight into factors promoting or

12   inhibiting trait loss. We tested whether a behavioural component of a sexual trait, male

13   calling effort, has been evolutionary reduced in silent populations of Hawaiian field crickets

14   (*Teleogryllus oceanicus*). Cricket song requires energetically costly wing movements, but

15   'flatwing' males have feminised wings that preclude song and protect against a lethal,

16   eavesdropping parasitoid. Flatwing males express wing movement patterns associated with

17   singing but, in contrast to normal-wing males, sustained periods of wing movement cannot

18   confer sexual selection benefits and should be subject to strong negative selection. We

19   developed an automated technique to quantify how long males spend expressing wing

20   movements associated with song. We compared calling effort among populations of

21   Hawaiian crickets with differing proportions of silent males, and between male morphs.

22   Contrary to expectation, silent populations invested as much in calling effort as non-silent

23   populations. Additionally, flatwing and normal-wing males from the same population did not

24   differ in calling effort. The lack of evolved behavioural adjustment following morphological

25   change in silent Hawaiian crickets illustrates how behaviour might sometimes impede, rather

26   than facilitate, evolution.

Introduction

A common intuition is that traits which no longer serve an adaptive function should be evolutionarily lost [1]. Secondary loss and vestigialisation of morphological traits are well-documented [2], but whether associated behaviours are also evolutionarily lost, for example through accumulation of neutral mutations [3,4], is less well understood. Alternatively, such behaviours could remain expressed and available for evolutionary co-option [5]. Secondary sexual traits provide an excellent opportunity to test this, because they frequently involve display behaviours that work in tandem with specialised morphological features to produce a conspicuous signal. The reduction and loss of sexual signals under natural selection is theoretically predicted [6,7] and widely observed [8,9]. Here we use populations of field crickets *Teleogryllus oceanicus* that have recently lost the ability to sing due to morphological change [10], yet retain central pattern generators that produce the behavioural component of song [11], to test whether selection has reduced the amount of effort males expend on non-functional signalling behaviour.

Male crickets produce song via movement of their forewings, causing sound-producing wing structures to resonate [12]. Females strongly prefer males that sing more [13–17], but calling behaviour incurs substantial energy expenditure [18–20] and is condition-dependent [15,21–23]. In *T. oceanicus*, males from populations on multiple Hawaiian islands have lost the ability to produce acoustic signals under selection from an acoustically-orienting parasitoid fly, *Ormia ochracea* [10]. In the best characterised example of song-loss, silence is caused by loss of sound-producing structures on the wings (the 'flatwing' phenotype). Flatwing's emergence and spread has been well-documented in several populations on different islands: Kauai (first observed in 2003), Oahu (2005), and Hawaii (2010) [10,24,27]. In both Kauai and Oahu, flatwing segregates as a single-locus X-linked trait [10,24]. Silent males are strongly disadvantaged in the context of sexual selection

52 [25,26], but the phenotype nevertheless spread rapidly under selection from the fly. Flatwing

53 males are capable of expressing the precise patterns of rhythmic forewing movement that

54 produce song in normal-wing males [11]. Given its energetic costs and other known life-

55 history trade-offs [15,18–23], calling effort – the amount of time spent producing wing

56 movements associated with calling song – should be selected against.

57   We developed an automated video analysis technique to assay calling effort of males

58 from populations with contrasting proportions of normal-wing and flatwing male phenotypes

59 [27]. Our primary goal was to test the prediction that silent populations would show evolved

60 reduction in calling effort compared with non-silent populations. We also estimated condition

61 and measured testes mass to evaluate whether calling effort is associated with proxy

62 measures of male quality. Finally, we tested whether, within a population, flatwing males

63 show lower calling effort than normal-wing males. Evidence consistent with our predictions

64 of reduced calling effort in silent populations would support the idea that behavioural

65 evolution has facilitated the rapid spread of adaptive silence in crickets by reducing costly,

66 fruitless singing effort. No difference in calling effort among populations or between morphs,

67 however, would suggest selection has not reduced this non-adaptive trait, and its persistence

68 could diminish the overall advantages of flatwing and enable co-option for other functions

69 [1].

70

71 **Methods**

72 Further detail is supplied in Supporting Information. We sampled sites on Kauai (K_{VL}: 100%

73 flatwing), Hawaii (H_{CL}: 90-100% normal-wing), and two nearby (~1km apart) sites on Oahu

74 with contrasting proportions of silent and non-silent males (O_{AC}: 100% flatwing; O_{CC}: ~50%

75 flatwing) in 2017 (Fig. 1A) [28]. Our estimation that ~50% of O_{CC} males would be able to

76 sing owing to normal-wing venation was revised down to ~25% following identification of

77 an additional silencing phenotype in this population ('curly-wing' [28]). No curly-wing males

78 were used in the current experiment. Assuming four generations per year, flatwing males had

79 been present in Kauai and Oahu populations for approximately 56 and 48 generations,

80 respectively, at the time of sampling; well within the ability of populations to exhibit adaptive

81 evolution [29–32]. Flatwing males have been observed in Hawaii since 2010, but at low

82 proportions (<10%) [24,27,28]. Lab stocks derived from each population were reared

83 identically for two generations to minimise field-based maternal effects.

84 All crickets were reared in the same incubator, and test subjects were isolated from

85 their final pre-adult instar. Upon adult eclosion we surgically removed the plectrum of the

86 right forewing, which is necessary to produce song, from normal-wing males (Fig. 1B), and

87 the same portion of the wing was also removed from flatwing males. All males were

88 therefore silent throughout their lives, avoiding confounds that could arise if males receive

89 auditory feedback from their own song or sing in response to others [33]. This enabled us to

90 test for constitutive, i.e. genetic, differences in calling effort between populations and

91 morphs. We tested experimental males once between 7 and 11 days post-adult eclosion;

92 previous studies have found cricket calling behaviour is repeatable [34,35] and heritable [36–

93 38]. We weighed males to the nearest 0.001g, measured pronotum lengths to the nearest

94 0.01mm, and calculated scaled mass index (SMI) as a proxy of body condition [39] using

95 pronotum length as the linear measurement.

96 We glued a reflective tag to the distal tip of each male's right forewing (Fig. 1B).

97 After a ca.12-hour recovery period, we filmed two-hour calling effort trials using a Nikon

98 D3300, recording under dim red light, with crickets physically and visually isolated from one

99 another. Trials began 15 minutes after onset of the dark cycle to coincide with peak calling

100 activity in wild populations [34]. Automated analysis was performed in MATLAB using

101 custom scripts to quantify the duration of wing movement bouts associated with calling song

102 for each cricket. Briefly, image brightness and contrast were adjusted and the background

103 removed so that only reflective wing tags were visible. Centre coordinates for each tag were

104 recorded so distances moved between frames could be calculated. Differences between X-

105 coordinates were used to determine whether each distance was positive or negative. Distances

106 were converted into the time/frequency domain using a continuous wavelet transform (CWT)

107 between 1 to 20 Hz. For wing movements to qualify as singing, four criteria needed to be

108 met: (1) mean power between 10-16 Hz (13 Hz being the observed frequency of *T. oceanicus*

109 wing movements during calling song, based on [11]) exceeded a threshold of 0.7 of the CWT

110 output; (2) 90% of the power in the CWT output was between 10-16 Hz; (3) peak frequency

111 power was between 9 and 17 Hz, and at this peak the CWT output power was greater than

112 1.5; (4) the duration of singing exceeded 0.6 seconds. Episodes of singing that restarted

113 within 10 seconds were recorded as a single bout. These parameters were chosen based on

114 careful analysis of singing in trials using unmuted normal-wing crickets (Supporting

115 Information). We validated the technique by tracking tagged, audible normal-wing males (*N*

116 = 22; 9h 34m of recording), and cross-referencing with simultaneous audio recording, which

117 indicated a low rate of error. We also used data from these recordings of unmuted normal-

118 wing males to confirm that the procedure of muting the muted normal-wing crickets used in

119 trials did not reduce their investment in calling effort compared to intact normal-wing males

120 (Supporting Information).

121　　　Statistical analysis was performed in R v.3.4.4. Calling effort (i.e. time spent singing,

122 [21–23]), was normalised by Yeo-Johnson transformation. Variation in calling effort was

123 analysed using a linear mixed model (LMM), with *population* modelled as a categorical

124 factor and *trial ID* as a random effect to account for batch effects. *Age* in days post-eclosion,

125 *total mass*, and *SMI* were included as covariates, scaled and centred, with *population* by

126 covariate interactions. Interactions which did not approach significance (P>0.1) were

127 removed from final models. Morph variation within mixed populations was analysed in the

128 same way, but with *morph* replacing *population* as the categorical variable. Visualisation

129 indicated a non-linear effect of SMI, so it was included as both a linear and quadratic

130 predictor using the R function *poly* to account for covariance. Models were tested with Type

131 III or II sums of squares depending on whether they did (III) or did not (II) include

132 interactions.

133

134 **Results**

135 Calling effort was recorded for 342 males (H_{CL}=95 [90 normal-wing, 5 flatwing]; O_{CC}=82

136 [45 normal-wing, 37 flatwing]; O_{AC}=85 flatwing, and K_{VL}=80 flatwing). O_{AC} and K_{VL}

137 populations reared from eggs collected from wild-caught females contained only flatwing

138 males, supporting the observation that normal-wing males are absent in these populations

139 [27,28,40].

140 Mean and median calling effort across populations were 458 and 137 (Q1: 44, Q3:

141 429) seconds, respectively. There was no evidence males from silent populations spent less

142 time calling (Table 1; Fig 1C). Similarly, there was no evidence in the O_{CC} population, in

143 which normal-wing and silent flatwing morphs co-occur, of any reduced investment in song

144 by flatwing males (Table S2) and there was a similar pattern in the H_{CL} population, though

145 with too few flatwing males for statistical comparison (Fig. 1D). Time spent calling scaled

146 positively with condition, but this relationship appeared to tail off at the highest measures of

147 condition (Fig. S1). Overall, the LMM accounted for little of the total variance in calling

148 effort (R^2=0.08). Including testes mass in the model as an additional covariate (reducing *N*

149 from 342 to 318) alongside somatic measures of mass and SMI produced similar results,

150 though with a significant interaction between mass and population, and testes mass did not

151 predict time spent calling (Table S1).

152
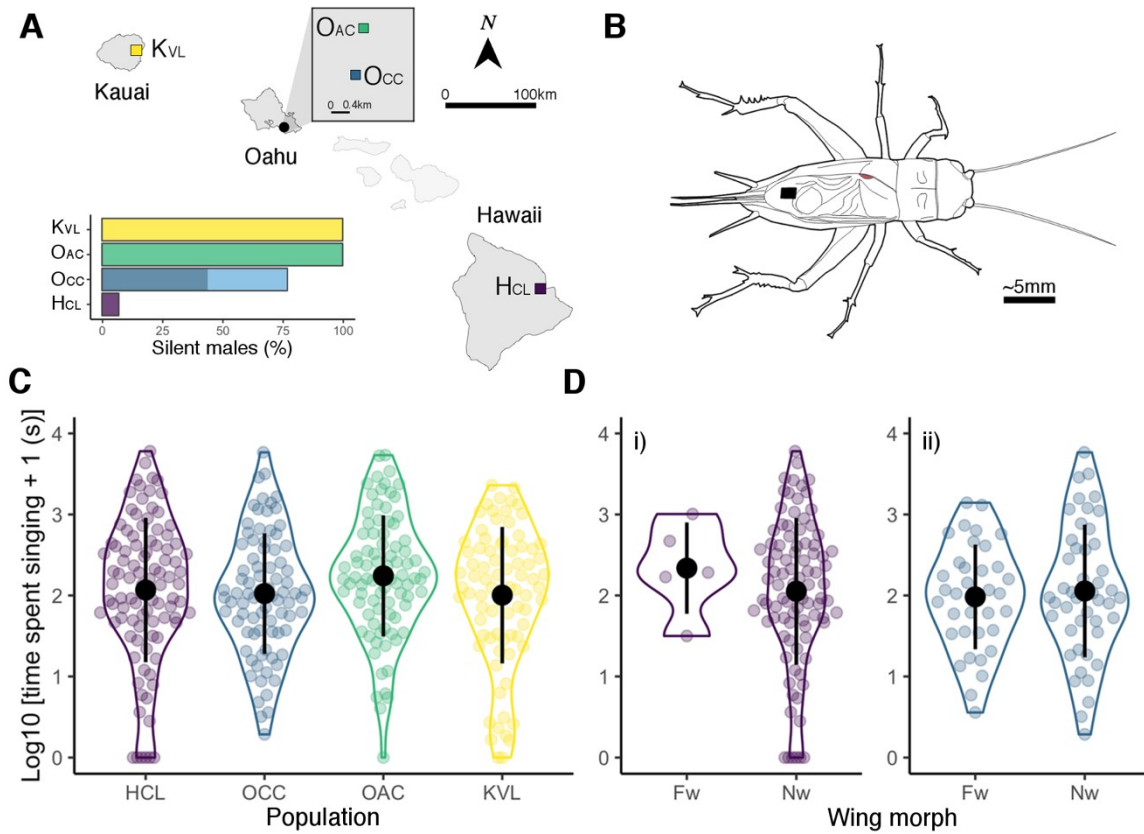
153

154 **Figure 1. A)** Map of the Hawaiian islands with locations of study populations. The inset

155 graph shows proportions of silent males in respective populations from [28]: the segmented

156 bar for O_{CC} illustrates ~33% of the silent males (the lighter portion) are silent due to curly-

157 wing rather than flatwing morphology. **B)** Diagram of a normal-wing male with a reflective

158 tag (black) applied to its dorsal-right forewing. The plectrum, surgically removed or sham-

159 operated in test males, is highlighted in red. **C)** Calling effort across populations, and **D)** of

160 Nw and Fw males in H_{CL} (i) and O_{CC} (ii) populations in which they co-occur; the H_{CL}

161 comparison is included for visualisation only and was not statistically tested due to the small

162 Fw sample. Points are jittered along X-axes, black points and error bars represent mean ± SD.

163

164 **Discussion**

165 Sexually selected traits are often opposed by countervailing natural selection [6,7,41,42],

166 which can lead to their evolutionary reduction or loss [8,9]. Understanding the consequences

167 for behaviours previously associated with the signal can provide information about factors

168 that may facilitate (if behaviour is lost) or stymie (if behaviour persists) such trait losses. We

169 found neither silent populations of *T. oceanicus*, nor silent males in mixed populations,

170 showed any reduction in calling effort. This calling effort will deplete energy reserves and

171 involve resource allocation trade-offs [19,20], reducing the relative fitness advantages of

172 silent males, and its persistence appears inconsistent with the view that behaviour facilitates

173 the early stages of adaptive evolution [43,44]. Here, persistent signalling behaviour imposes a

174 double cost on flatwing males: not only can they not produce signals, they still expend effort

175 trying to do so.

176 Evolved song loss in Hawaiian populations of *T. oceanicus* has been well-

177 documented, and has occurred through a variety of morphological means [10,24,28]. In all

178 cases, males which cannot produce song at ordinary levels persist in attempting to sing

179 [11,28]. It is perhaps unsurprising that silent flatwing males exhibit the same calling effort as

180 normal-wing males: for flatwing crickets to evolve lower song effort than normal-wing

181 crickets from the same population would require that mutations causing reduced investment

182 become genetically linked with causative *flatwing* mutation(s) on the X-chromosome, which

183 is unlikely. However, the persistence of calling effort in populations where all or almost all

184 males are silent, and have been for many generations, is surprising. Calling effort appears to

185 be an evolvable trait in field crickets; evolutionary shifts in the onset of calling have been

186 documented in *T. oceanicus* [45], and several cricket species are behaviourally mute [46]. It

187 remains an open question why morphological losses of sexual signalling in this system have

188 not been accompanied by vestigialisation of associated signalling effort.

189   The persistence of costly calling behaviours in silent males and in silent populations

190   of *T. oceanicus* is not without parallel in other species. Populations of rattlesnakes of the

191   genus *Crotalus* express only vestigial rattles and are incapable of producing a warning signal,

192   but nevertheless silently 'rattle' their tails when disturbed by humans [47–49]. Along similar

193   lines, several species of the *adiastola* group of Hawaiian *Drosophila* exhibit a courtship ritual

194   in which they raise and vibrate their abdomen, yet males of only one species express long

195   clavate hairs which sweep the female's head as this occurs. The elaboration of these hairs is

196   suggested to have evolved following the courtship behaviour, rather than having been lost in

197   related lineages [50]. It is plausible that if selection against song-associated wing movements

198   in Hawaiian field crickets is sufficiently weak, these movements could be similarly co-opted,

199   for example via substrate-borne vibrations detectable at close-range [51], or lead to the regain

200   of song [40,52]. Gray et al. [52] demonstrated the feasibility of the latter scenario by inducing

201   expression of calling song in a cricket species, *Gryllus ovisopis*, in which it has been

202   evolutionarily lost.

203   Our results suggest an interesting counterpoint to the widely supposed role of

204   behaviour in the early stages of rapid adaptation [43,44]; constraints associated with less

205   evolutionarily responsive behaviours might reduce the benefit of novel adaptive variants. In

206   the case of song-loss in *T. oceanicus* these costs are evidently marginal compared with the

207   overwhelming benefits of evading parasitism, but under different conditions, similar costs

208   could inhibit or even preclude populations from rapidly adapting to changes in their

209   environment. This maintenance of 'vestigial behaviours' could play an important role in

210   eventual re-emergence of previously lost traits over longer-term evolutionary timescales

211   [40,53].

212

213 **Acknowledgements**

217

Data accessibility

219   Raw data and scripts are available as supplementary material. Raw data have also been

220   deposited at https://doi.org/10.5061/dryad.g79cnp5m8 (pending activation)

221

222   Temporary URL: https://datadryad.org/stash/share/qMJl_uP-

223   xKQZMp1jTvCQZ6hkCGEL06g_KMHR1hDwy10

224

Author contributions

226   JGR, WTS & NWB conceived experiments; WTS & JGR implemented experimental design;

227   JGR & NWB performed fieldwork; JGR conducted experiments; WTS automated video

228   tracking; JGR performed statistical analysis; JGR & NWB wrote the manuscript with input

229   from WTS. All authors approve the final version of the manuscript and agree to be held

230   accountable for the work therein.

231

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378

379 **Tables**

380 **Table 1.** The results of an LMM (N=342, R^2=0.08) for time spent singing, with a random

381 effect of trial.

382

Predictor	df	X^2	P
Population	3	2.119	0.548
Age	1	0.067	0.798
Mass	1	0.589	0.443
SMI[*]	2	18.583	**<0.001**
Population × mass	3	6.559	0.087

383 Significant P-values (<0.05) are highlighted in bold.

384 * SMI is included in the model as an orthogonal polynomial with 2 degrees, to account for non-linear

385 effects